

Abscisic Acid-Responsive Protein, Bovine Serum Albumin, and Proline Pretreatments Improve Recovery of *in Vitro* Currant Shoot-Tip Meristems and Callus Cryopreserved by Vitrification

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Improved recovery of vitrified currant (*Ribes aureum* Pursh and *R. ciliatum* Humb. & Bonpl.) meristems and callus was obtained following 2 h pretreatment in sucrose, proline, abscisic acid-responsive proteins (RABP), or bovine serum albumin (BSA). Two hours immersion in 0.4 M RIB-SM prior to vitrification greatly improved the regrowth of meristems compared to 0, 1, 3, and 4 h immersion. Two hours immersion of meristems in 5 and 10% proline dissolved in 0.4 M RIB-SM significantly improved regrowth following vitrification. Initial tests with extracts of crude RABP from wheat seeds showed that regrowth of vitrified *Ribes* apical meristems improved after 2 h immersion pretreatment with the highest survival at 1% RABP. RABP preparations containing equivalent proteins (1% crude or 0.2% dialyzed RABP) had similar effects on regrowth, indicating that the effect was from the proteins rather than sugars and other carbohydrates in the crude RABP extracts. Pretreatments of meristems and callus with 5 or 10% proline, 1% crude RABP, or 1% BSA in 0.4 M sucrose solutions produced similar results. Pretreated meristems resumed growth 3 days after warming and reached the maximum regrowth at 1 week, compared to 2 weeks for non-pretreatment controls. We suggest using a 1% BSA pretreatment as the most economical and available of the materials tested. © 1997 Academic Press

Cryopreservation as a long-term storage method for germplasm has developed rapidly over the past decade. The development and modification of cryopreservation protocols are important to successfully preserve a broader range of plant species and cultivars. Pretreatments may be used to condition cells and tissues to withstand cooling and the toxic effects of cryoprotectant solutions.

Cryoprotectants, used to lower the homogeneous nucleation temperature, slow the rate of crystal growth, and increase glass transformation temperature (18), may also be a source of cellular injury. Toxic effects of cryoprotectants [glycerol, dimethyl sulfoxide (Me₂SO)] used in slow-freezing protocols are minimized by pretreatment with sugars, sugar alcohols, and amino acids introduced in solid or liquid medium (20, 33). Pretreatment reduces the cell size and the cytoplasm to vacuole ratio,

enhances the ability of cells or tissues to take up cryoprotectants during prolonged exposure, and/or modifies cell walls and membranes to resist dehydration injury and deformation during freezing (33). Sucrose and proline stabilize membrane bilayers and enzymes during desiccation and freezing (3, 6, 30). They also act in a colligative manner to prevent a toxic level of compounds from accumulating in membranes during dehydration and freezing. These colligative cryoprotectants must be penetrating and present at high concentrations to be effective (11, 32).

Vitrification, a newly developed cryopreservation protocol, uses highly viscous solutions or extreme desiccation to form a glass at low temperature and is now used to preserve plant cells and tissues in liquid nitrogen (LN). The available solutions required for vitrification are highly toxic, and excessive exposure may damage membrane integrity, inhibit photosynthesis, cause severe plasmolysis, and predispose cells to deplasmolysis injury (17, 27, 34). Present vitrification protocols use

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only limited pretreatment regimes, and modifications are needed to improve regrowth.

The natural cryoprotective mechanisms present in many seeds and plants may provide a new source of pretreatment chemicals. New proteins are synthesized following changes in gene expression in response to stresses such as cold or dehydration. Among these proteins are abscisic acid-responsive proteins (RABP), including late embryogenesis abundant (LEA) proteins, some cold-regulated (COR) proteins, and dehydration-inducible proteins (dehydrin, DHN) (2, 7, 9, 13). These proteins are hydrophilic and heat-stable. The protein COR15 was more effective on a molar basis in protecting lactate dehydrogenase (LDH) against freeze inactivation *in vitro* than sucrose or bovine serum albumin (BSA) (15). Cryoprotective effects include protecting isolated chloroplast and thylakoid membranes against mechanical rupture by reducing solute permeability during freezing, increasing expandability during warming, and increasing resistance against the osmotic stress (10, 12). RABP, like other cryoprotective proteins, are probably noncolligative in action and may affect specific loci of the outer membrane (32). RABP may act as hydrating agents; they may also bind excess ions to prevent cytotoxicity due to increased ion concentration during desiccation (1). These properties of RABP may make them useful as pretreatments or cryoprotectants for cells or tissues.

The effect of RABP on the recovery of cryopreserved meristems and callus has not been previously studied. The objective of this research was to investigate the effectiveness of an RABP extract from wheat seeds as a pretreatment agent to improve the recovery of cryopreserved meristems and callus. In addition, we compared the effectiveness of RABP with that of sucrose, proline, and BSA. Sucrose was tested as a pretreatment because it is a major component of the base medium for plant vitrification solutions, proline because it is successfully used in other pretreatment methods, and BSA because it is a readily available protein. RABP used in this research were extracted from wheat seeds (23, 24).

MATERIALS AND METHODS

Plant Materials

Ribes aureum Pursh (Rib 125) and *R. ciliatum* Humb. & Bonpl. (Rib 670) *in vitro* plantlets were provided by the USDA/ARS National Clonal Germplasm Repository (NCGR) at Corvallis, Oregon, U.S.A.

General Growth Conditions

Plantlets. *In vitro* *Ribes* (currant) plantlets from stock cultures were grown in Magenta GA7 (Magenta Corp., Chicago, IL, U.S.A.) plastic boxes with 50 ml of NCGR-RIB growth medium (RIB-GM) composed of MS salts and vitamins (19) with 30% of the normal ammonium and potassium nitrate concentrations, pH 5.7, with (per liter) 50 mg ascorbic acid, 20 g glucose [D-(+)-glucose], 0.1 mg N⁶-benzyladenine (BA), 0.2 mg gibberellic acid (GA₃) (Sigma, St. Louis, MO, U.S.A.), 3.5 g agar (Bitek agar, Difco, Detroit, MI, U.S.A.), and 1.45 g Gelrite (Kelco, San Diego, CA, U.S.A.). All medium components were autoclaved. Twenty-five plantlets were cultured in each box and transferred every 3 weeks. Plantlets were grown for 2 weeks after transfer before use in experiments. Growth room conditions were 16-h photoperiod (25 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at 25°C.

Callus. Stems of *in vitro* grown *R. aureum* and *R. ciliatum* were cut into segments (2–3 mm) and grown for callus in GA7 boxes with 50 ml of NCGR-RIB medium salts and vitamins as for plantlets and (per liter) 0.04 mg BA, 1 mg indole-3-butyric acid (IBA), and 1 mg 2,4-dichloro-phenoxyacetic acid (2,4-D) (Sigma). Twenty-five callus cultures (4–5 mm in diameter) were induced and cultured in the dark at 25°C and transferred every 2 weeks. Pale yellow callus (10 days) was selected and used for experiments.

Cold Acclimatization (CA)

After 2 weeks (for plantlets) or 10 days (for callus) in the medium under general growth conditions, the boxes (25 plantlets or calli/box) were moved into a CA chamber for 1

week. CA was 22°C for 8 h ($3 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) and -1°C for 16 h (dark) (22).

Preculture of Meristems and Callus

After cold acclimatization, 25 meristems (0.8 mm) were dissected and precultured on RIB-GM with 5% dimethyl sulfoxide (Me_2SO , Sigma), 3.5 g agar, and 1.75 g Gelrite. Twenty-five calli were also transferred to Me_2SO medium. All meristems and callus remained under CA conditions on Me_2SO medium for 48 h (14).

Pretreatment Regimes

Sucrose liquid medium: Determination of immersion time. Preliminary experiments were run to determine optimum pretreatment times for 0.4 M RIB-SM. These treatment times were then used for the protein and proline pretreatments. Twenty-five meristems for each treatment were transferred to 1.2-ml cryovials (Vanguard Cryos, Sumitomo Bakelite Co., Ltd., Japan) with 1 ml 0.4 M sucrose in liquid NCGR-RIB medium (0.4 M RIB-SM) and immersed for 1, 2, 3, and 4 h. Twenty meristems were vitrified by plunging into LN (see below), and 5 were used for nonvitrified controls with three replications ($n = 60$). As a treatment control, a nonimmersed group (0 h pretreatment) was vitrified directly following the pregrowth phase.

Proline, crude and dialyzed RABP, and BSA pretreatment. Meristems or callus tissues were immersed in 1 ml of pretreatment solutions for 2 h and vitrified in LN (20 meristems, 15 calli) or used for nonvitrified controls (5 meristems, 10 calli). Pretreatments included 2 h immersion in 0.4 M RIB-SM alone or with one of the following: 1 (w/v), 5, 10, and 15% proline solutions; 0.5, 1, and 2% crude RABP; 0.2% dialyzed RABP; and 1% BSA (Fraction V, 15.9% Nitrogen, Sigma). Proline, RABP (crude and dialyzed), and BSA were dissolved in 0.4 M RIB-SM and filter-sterilized (25 mm, 0.20 μm , Corning Glass Works, Corning, NY, U.S.A.). Crude and dialyzed RABP, prepared from wheat seed, were provided by Dr. M. K. Walker-Simmons (USDA/ARS, 209 Johnson Hall, Washington State University, Pullman,

WA, U.S.A.) (24). RABP were dialyzed in Spectra/Por (Fisher Scientific) tubing (molecular weight cutoff of 12,000–14,000). The dry weight of dialyzed RABP was about 20% of the original extracted material.

Vitrification Procedure

General vitrification procedures for meristems were identical to those described by Reed and Yu (22). Pretreatment solutions were replaced with PVS2 (plant vitrification solution #2 composed of 15% Me_2SO , 15% ethylene glycol, and 30% glycerol, brought to volume with 0.4 M RIB-SM) (27). The solution was replaced twice, ending with a final volume of 1 ml PVS2 per cryotube. Samples were incubated in PVS2 on ice for 20 min. The optimal incubation duration of callus in PVS2 (20 min) was determined in preliminary experiments by holding samples in PVS2 on ice for 5, 10, 15, 20, and 25 min before vitrification. The cryovials were plunged directly into LN and exposed for at least 1 h. Meristems or callus in control groups (without pretreatment) was transferred directly to 1.2-ml cryovials with PVS2 and held for 20 min on ice. The controls were immediately recovered from PVS2 solution and plated as described below.

Cryovials were removed from the LN dewar, immediately plunged into 45°C water for 1 min, and transferred to 22°C water for another min. After warming, PVS2 solution in the cryovials was immediately diluted with 0.2 ml rinsing medium (1.2 M sucrose in liquid NCGR-RIB medium), the solutions were removed, and meristems and callus were rinsed three times in the diluting medium. Meristems were drained on filter paper strips (Whatman International Ltd., Maidstone, England) and plated in individual wells of a 24-well plate (Costar Corp., Cambridge, MA, U.S.A.) containing RIB-GM. All the meristems were grown under the same general conditions as for plantlets, except that the first week of growth was in the dark. Regrowth of meristems was recorded as the percentage of green and growing shoots 4 weeks after warm-

ing. Callus was held in rinsing medium (1.2 M liquid NCGR-RIB medium) for 5 min followed by the viability test procedures described below.

Viability Test for Callus

A modified TTC (triphenyl tetrazolium chloride) reduction assay (5) was used to determine viability. Thawed callus was rinsed twice in 1% TTC solution in potassium phosphate buffer, pH 5.8, then 1% TTC solution was added to an equal volume of callus. The cryovials with callus and TTC solution were held overnight at room temperature in the dark. Living cells turned red.

After staining, the callus was rinsed twice in deionized filtered water and placed in 3 ml 100% ethyl alcohol (Nalge Co., Rochester, NY, U.S.A.). The red pigment was extracted at 70°C for 30 min. Absorbance was read at 530 nm (Spectronic 20, Bausch & Lomb Inc., Rochester, NY, U.S.A.). The viability of callus after warming was expressed as

Relative percentage survival (%)

$$= \frac{A_{530}(\text{vitrified})}{A_{530}(\text{control})} \times 100.$$

Statistical Analysis

The percentage meristem regrowth or percentage survival for callus was transformed to arcsin and analyzed with ANOVA and the Duncan's multiple range test with significance designated as $P \leq 0.05$ using STATGRAPHICS 5.0 (Statistical Graphics Corp. and STSC Inc., Rockville, MD, U.S.A.).

RESULTS

Meristems

Immersion time in 0.4 M RIB-SM. Pretreatment of *R. ciliatum* meristems in 0.4 M RIB-SM for all four immersion times before PVS2 exposure and vitrification produced more regrowth than direct PVS2 exposure alone (Table 1). Meristems immersed for 2 h produced the highest regrowth and those with no pretreatment had the lowest. There were no sig-

nificant differences among the regrowth of meristems with 1, 3, and 4 h immersion ($P < 0.05$). Extended immersion (3 and 4 h) in the 0.4 M RIB-SM significantly decreased meristem regrowth, compared to the 2-h pretreatment.

Proline pretreatment. Using the optimum pretreatment times determined for the 0.4 M RIB-SM pretreatment, added proline was found to significantly increase the regrowth of meristems compared to sucrose alone. Proline (5 and 10%) added to the 0.4 M RIB-SM pretreatment solution significantly improved the regrowth of vitrified *R. ciliatum* meristems ($P < 0.05$) compared to pretreatment with 0.4 M RIB-SM alone or with 1% proline, 15% proline, and non-pretreatment (Table 2). There were no significant differences in regrowth among meristems pretreated with 0.4 M RIB-SM alone or with 1 and 15% proline solution or between pretreatment solutions with 5 and 10% proline ($P < 0.05$).

Pretreatment with Crude and Dialyzed RABP and BSA Solutions

Comparison of crude RABP concentrations. Pretreatment of *R. ciliatum* meristems in 0.4 M RIB-SM with 1% (w/v) and 2% crude RABP significantly improved regrowth ($P < 0.05$), compared to meristems without pretreatment or with pretreatments of 0.4 M RIB-SM alone and with 0.5% crude RABP (Table 3). Regrowth of meristems pretreated with solutions including 1% crude RABP was significantly better than those with 2% and gave the best regrowth of all pretreatments. There was no significant difference between pretreatment with 0.4 M RIB-SM alone and with 0.5% crude RABP.

Comparison of proline, crude and dialyzed RABP, and BSA solutions. Both crude and dialyzed RABP pretreatment produced significant improvement in regrowth of cryopreserved meristems of *R. ciliatum* and *R. aureum* following vitrification, compared to pretreatment with 0.4 M RIB-SM or non-pretreatment ($P < 0.05$) (Table 4). There was no significant

TABLE 1
Optimum Immersion Time for Pretreatment of *Ribes ciliatum* Meristems in 0.4 M Sucrose
in Liquid Growth Medium (0.4 M SM)

Replication	Percentage regrowth ^a				
	Immersion pretreatment (hr)				
	None	1	2	3	4
1	42.13	45	56.79	50.77	47.87
2	39.23	47.87	53.73	50.77	50.77
3	42.13	47.87	53.73	47.87	47.87
Mean ^b	41.16 c	46.91 b	54.75 a	49.80 b	48.84 b
Standard error (SE)	0.97	0.96	1.02	0.97	0.97
Source of variation ^c	Mean square	F ratio			
Between groups	72.75	25.48*			
Within groups	2.86				

^a Percentage regrowth data were transformed by $\arcsin \sqrt{X}$. Four weeks after thawing.

^b Mean separation by Duncan's multiple range test at $P \leq 0.05$ ($n = 60$). Values in a row with different letters (a-c) are significantly different.

^c Variation between treatments: four degrees of freedom.

* Significant at $P \leq 0.001$.

difference between 1% crude and 0.2% dialyzed RABP ($P < 0.05$).

Significant improvement in regrowth of cryopreserved meristems of both species following vitrification was obtained with pretreatment solutions containing 5% proline, 1% crude RABP, 0.2% dialyzed RABP, and 1% BSA, compared to that of 0.4 M RIB-SM alone and the non-pretreatment control ($P < 0.05$). There were no significant differences among 5% proline, 1% crude RABP, 0.2% dialyzed RABP, and 1% BSA solutions ($P < 0.05$).

Time course of regrowth of vitrified meristems. Three days after warming and plating, most of the meristems of *R. ciliatum* resumed growth (Fig. 1). Shoots grew directly from meristems without a callus phase. The meristems in most of the pretreatment groups reached maximum regrowth after 1 week, while the non-pretreatment control required 2 weeks. Among control groups, pretreated meristems regrew faster than those exposed to PVS2 without pretreatment (data not shown).

Callus

Pretreatment with solutions containing 5% proline, 1% crude RABP, and 1% BSA significantly improved the survival of cryopreserved *R. ciliatum* and *R. aureum* callus following vitrification, compared to 0.4 M RIB-SM alone and non-pretreatment ($P < 0.05$) (Table 5). There were no significant differences in survival among pretreatments with 5% proline, 1% crude RABP, and 1% BSA ($P < 0.05$). Regrowth of callus tissues was not studied.

DISCUSSION

The sucrose pretreatment produced expected increases in survival following vitrification. Sucrose is known for its natural cryoprotectant qualities because it accumulates in some organisms following cold or drought stress (8). Sakai and Yoshida (26) found that sucrose was one of the best cryoprotectants for cabbage cells. Sucrose serves as an osmotic agent in cells and protects membranes by stabilizing enzymes and membrane bi-

TABLE 2
Optimum Immersion Time for Pretreatment of *Ribes ciliatum* Meristems in 0.4 M Sucrose in Liquid Growth Medium (0.4 M SM) Alone or with 1, 5, 10, or 15% Proline

Replication	Immersion pretreatment (% regrowth) ^a					
	None	0.4 M SM	0.4 M SM combined with proline			
			1%	5%	10%	15%
1	45	53.73	50.77	63.43	63.43	53.73
2	47.87	53.73	53.73	71.57	67.21	56.79
3	50	56.79	53.73	67.21	63.43	56.79
Mean ^b	45.96 c	54.75 b	52.74 b	67.40 a	64.69 a	55.77 b
Standard error (SE)	0.96	1.02	0.99	2.35	1.26	1.02
Source of variation ^c	Mean square	F ratio				
Between groups	188.36	33.98*				
Within groups	5.54					

^a Percentage regrowth data were transformed by $\arcsin \sqrt{X}$. Four weeks after thawing.

^b Mean separation by Duncan's multiple range test at $P \leq 0.05$ ($n = 60$). Values in a row with different letters (a-c) are significantly different.

^c Variation between treatments: four degrees of freedom.

* Significant at $P \leq 0.001$.

layers. Pretreatment with sucrose solutions reduces the amount of cellular water by osmosis and is shown to improve the survival of cryo-

preserved somatic embryos (6). Our studies confirmed that sucrose immersion pretreatment protected cells and tissues and improved

TABLE 3
Optimum Immersion Time for Pretreatment of *Ribes ciliatum* Meristems in 0.4 M Sucrose in Liquid Growth Medium (0.4 M SM) Alone or with 0.5, 1, or 2% Crude RABP

Replication	Immersion pretreatment (% regrowth) ^a				
	None	0.4 M SM	0.4 M SM combined with crude RABP		
			0.5%	1%	2%
1	42.13	53.73	56.79	63.43	60
2	45	56.79	60.00	67.21	63.43
3	42.13	53.73	56.79	67.21	63.43
Mean ^b	43.09 d	54.75 c	57.86 c	65.95 a	62.29 b
Standard error (SE)	0.96	1.02	1.07	1.26	1.14
Source of variation ^c	Mean square	F ratio			
Between groups	230.41	64.05*			
Within groups	3.60				

^a Percentage regrowth data were transformed by $\arcsin \sqrt{X}$. Four weeks after thawing.

^b Mean separation by Duncan's multiple range test at $P \leq 0.05$ ($n = 60$). Values in a row with different letters (a-d) are significantly different.

^c Variation between treatments: four degrees of freedom.

* Significant at $P \leq 0.001$.

TABLE 4

Effect of Pretreatments on the Regrowth of Vitrified *Ribes aureum* and *R. ciliatum* Meristems 4 Weeks after Warming: 0.4 M Sucrose in Liquid Medium (0.4 M SM) Alone or with 5% Proline, 1% Crude RABP (cRABP), 0.2% Dialyzed RABP (dRABP) or 1% BSA

Genotype	Immersion pretreatment (% regrowth) ^a					
	None	0.4 M SM combined with				
		0.4 M SM	5% Proline	1% cRABP	0.2% dRABP	1% BSA
<i>R. aureum</i>	44.04 ± 0.96 c	52.74 ± 0.99 b	64.69 ± 1.26 a	65.95 ± 1.26 a	67.40 ± 2.35 a	68.66 ± 1.45 a
<i>R. ciliatum</i>	42.13 ± 0 c	53.76 ± 1.74 b	67.40 ± 2.35 a	76.26 ± 6.98 a	68.86 ± 2.71 a	67.55 ± 2.49 a
Source of variation ^b	<i>R. aureum</i>			<i>R. ciliatum</i>		
	Mean square		F ratio	Mean square		F ratio
	295.50		46.51*	462.26		13.05*
Between groups	6.35			35.44		
Within groups						

^a Percentage regrowth data were transformed by arcsin \sqrt{X} and expressed as means ± SEM%. Mean separation by Duncan's multiple range test at $P \leq 0.05$ ($n = 60$). Values in a row with different letters (a-c) are significantly different.

^b Variation between treatments: five degrees of freedom.

* Significant at $P \leq 0.001$.

the recovery of meristems during the cooling-rewarming cycle. Two hours immersion in the sucrose solution was optimal for *Ribes* meristems and callus to adsorb adequate sucrose molecules for effective protection without adverse effects. However, extended immersion (3 and 4 h) of meristems prior to vitrification resulted in decreased regrowth. Extended immersion in sucrose solutions may overly dehydrate tissues or cells or may exclude oxygen from the tissues, causing injury unrelated to the effects of low temperature.

Proline, another natural cryoprotective substance produced following stress, is one of the cryoprotective amino acids which functions through a colligative effect and interacts with cell membranes at specific loci (11) and may act by a preferential exclusion mechanism at lower temperatures (3). Proline inhibits membrane mixing during freezing and stabilizes bilayers by preventing the aggregation of intramembranous particles (25). Postthaw viability of *Zea mays* suspension cultures increased with a 3- to 4-day pretreatment of 10% proline (w/v) in the medium (33). Precul-

ture on medium with 5% Me₂SO and 5% proline improved the recovery of cryopreserved peach meristems (21). Our results indicated that 2 h immersion in 5 or 10% proline dissolved in 0.4 M RIB-SM improved the recovery of vitrified *Ribes* meristems and callus. Increasing the concentration of proline to 15% significantly decreased the regrowth. With *Z. mays* L. cells it is suggested that the permeability of cell membranes is altered by proline, allowing better uptake of cryoprotectants (33). Improved PVS2 uptake due to improved cell permeability could account for improved survival of *Ribes* meristems after pretreatment.

Cryoprotective proteins or antifreeze proteins (AFP) from both plants and fish are shown to protect plant enzymes, membranes of organelles, cells, and leaves (4, 12, 15). In this study, pretreatment with RABP from wheat seeds was effective in protecting meristems and callus against the toxic effects of vitrification solutions and possibly the effects of low temperature. Preliminary experiments indicated that RABP alone were ineffective as a cryoprotectant or when added to PVS2 (data

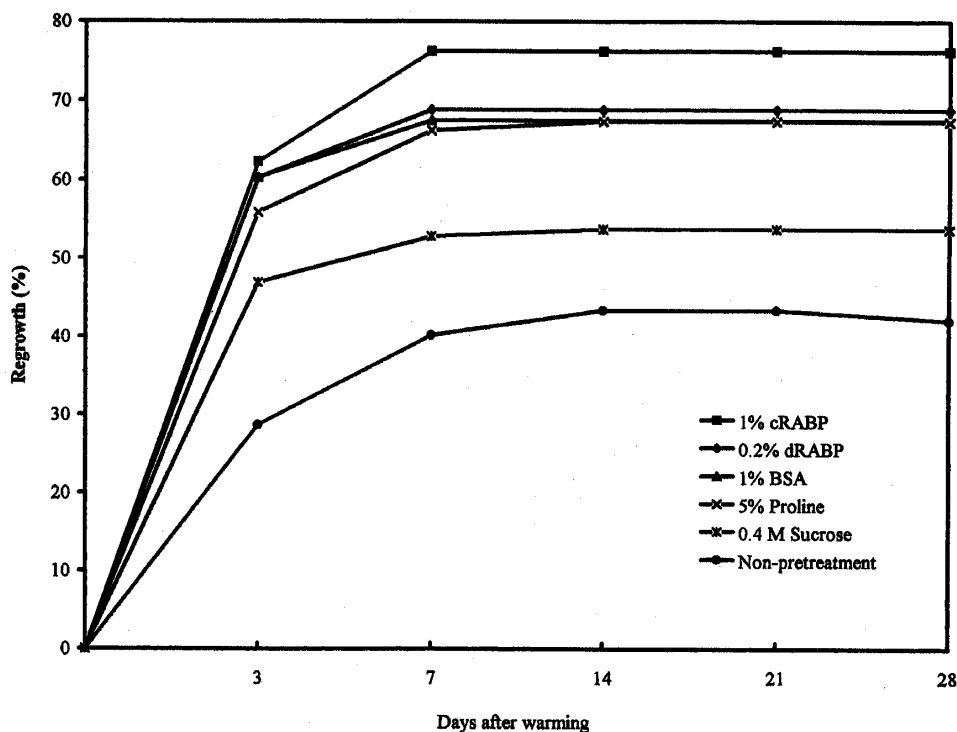


FIG. 1. Time course of regrowth of meristems of *R. ciliatum* Humb. & Bonpl. following 2-h pretreatments in 0.4 M RIB-SM alone or with 5% proline, 1% BSA, 0.2% dialyzed RABP (dRABP), and 1% crude RABP (cRABP) or non-pretreated control, vitrification in PVS2, and rapid warming ($n = 60$).

not shown), but significantly improved regrowth of meristems when followed by PVS2 cryoprotection. A significant improvement in recovery of vitrified meristems and callus was obtained from pretreatment with 1% crude RABP or 0.2% dialyzed RABP, indicating that effectiveness was from the proteins rather than the carbohydrates present in crude RABP extracts (Tables 4 and 5). Because of their hydrophilic character, RABP may bind water molecules from cells or tissues, and, because of their high molecular weight (10–100 kDa), RABP probably do not penetrate into cells, but perhaps attach to, or coat, cell and tissue surfaces. The mode of action of RABP in seedlings, protecting them from desiccation damage, is proposed to be as an ion carrier which prevents the precipitation or crystalliza-

tion of ions inside cells of drying tissues (7). Initially, RABP appeared promising as a cryoprotectant due to its effects in wheat seedlings. We do not know if the positive effects of RABP on the survival and regrowth of *Ribes* meristems are due to binding of ions in the cell, osmotic effects, or some action resulting in detoxification of some component of PVS2. The presence of RABP, like sucrose and proline, may modify cell structures and stabilize membranes, preparing them for dehydration and vitrification (34).

High RABP concentrations (2%) in pretreatment solutions decreased regrowth of vitrified *Ribes* meristems when compared to the 1% pretreatment. High BSA concentrations (2 to 3%) in cryoprotectant solutions damaged isolated thylakoid membranes, but the damage

TABLE 5

Effect of Pretreatments on the Survival of Vitrified *Ribes aureum* and *R. ciliatum* Callus as Determined by the TTC Reduction Test: 0.4 M Sucrose in Liquid Medium (0.4 M SM) Alone or with 5% Proline, 1% Crude RABP (cRABP), or 1% BSA

Genotype	Immersion pretreatment (% Survival) ^a				
	None	0.4 M SM	0.4 M SM combined with		
			5% Proline	1% cRABP	1% BSA
<i>R. aureum</i>	39.84 ± 0.52 c	45.80 ± 0.42 b	50.60 ± 0.84 a	51.00 ± 0.62 a	50.56 ± 0.46 a
<i>R. ciliatum</i>	39.92 ± 2.12 c	48.74 ± 1.23 b	60.98 ± 0.75 a	64.30 ± 1.89 a	64.21 ± 2.16 a
Source of variation ^b	<i>R. aureum</i>		<i>R. ciliatum</i>		
	Mean square		Mean square		F ratio
	F ratio		F ratio		
Between groups	69.57		353.88		39.64*
Within groups	1.05		8.93		

^a Percentage survival data were transformed by arcsin \sqrt{X} and expressed as means ± SEM%. Mean separation by Duncan's multiple range test as $P \leq 0.05$ ($n = 45$). Values in a row with different letters (a-c) are significantly different.

^b Variation between treatments: four degrees of freedom.

* Significant at $P \leq 0.001$.

could be avoided by adding high concentrations of ethylene glycol monomethyl ether prior to freezing to -20°C (28).

Because RABP is a mixture of groups of proteins, the separation of protein fractions will be necessary to further investigate whether the positive effect is from the mixture of proteins or from one or several protein combinations. We did not determine the extent of RABP infiltration into the tissues. Vacuum infiltration and quantitation of radioactive RABP will be required to determine how much RABP was absorbed or adsorbed to meristems and callus, and the relationship to cryoprotective effects would be more clearly illustrated.

BSA is often applied in animal cell culture as a growth factor (16). In plants the addition of BSA maintains plasma membrane integrity during protoplast dehydration (30) and protects LDH activity against freeze denaturation (31). BSA is sometimes used in cryoprotectant mixtures at higher concentrations (6%) (29). Our study with *Ribes* showed improved recovery of cryopreserved meristems and callus

with 2 h pretreatment with 1% BSA (Tables 4 and 5). The mechanism of BSA protection may be the same as that of RABP because BSA also has a high molecular weight (66 kDa) and stabilizes membrane structure.

We found no significant differences in effectiveness between RABP and BSA. The mode of functioning of RABP proteins is not well understood, and similarly the effects of BSA on plant cells are not widely studied. BSA had not been used as a pretreatment in earlier studies; rather, it was used as part of a cryoprotectant solution. The beneficial effects of BSA in this study indicate that it may exert its influence more effectively when applied earlier in the cryoprotection scheme.

An unanticipated result of these pretreatment protocols was the rapid recovery of meristems in almost all pretreatment groups (Fig. 1). Meristems from most pretreatment groups resumed growth 3 days after rewarming and reached their maximum recovery at 1 week, while 2 weeks was required for vitrified, non-pretreatment controls. Even pretreated controls (not vitrified) recovered faster than those

without pretreatment (data not shown). The rapid recovery of pretreated meristems indicated the effectiveness of all the pretreatments (sucrose, proline, RABP, and BSA) in protecting meristems and callus against cryoprotectant injury. Cryoprotectant injury to *Ribes* meristems is common with highly concentrated vitrification solutions such as PVS2 (22), but was overcome with these pretreatments.

Many factors, including pretreatment, affect the recovery of vitrified meristems and callus tissues. Our present research provides a simple and effective pretreatment method which adds a 2-h immersion of specimens to the standard vitrification procedure. Immersion facilitates the absorption into or adsorption of pretreatment solutions onto the specimens. Several of the pretreatment solutions improved the survival of vitrified meristems.

We suggest the use of BSA as a pretreatment for *Ribes* meristems due to its wide availability and low cost. Proline and RABP are equally useful, but more expensive and, in the case of RABP, not readily available. This pretreatment scheme may also be useful with other species or other cryopreservation techniques.

CONCLUSIONS

Pretreatment with 2 h immersion was an effective procedure to improve recovery of vitrified meristems and survival of callus of two currant species, *Ribes aureum* and *R. ciliatum*. Pretreatment solutions with 5% proline, 1% crude RABP, 0.2% dialyzed RABP, or 1% BSA in 0.4 M RIB-SM significantly improved the regrowth of cryopreserved meristems and callus, compared to pretreatment with 0.4 M RIB-SM alone or no pretreatment. The pretreatment solutions were probably effective by different mechanisms, including osmotic effects for sucrose solutions; colligative effects for proline and sucrose; and noncolligative effects for proline, RABP, and BSA. Finally, all these solutions play an important role in maintaining the integrity of cell structures and stabilizing cell membranes during the cool-

ing-rewarming cycle, and this results in improved recovery following warming.

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